Electro-generated Chemiluminescent Determination of Methotrexate in Pharmaceutical Preparations by Tris (2,2'-bipyridine)ruthenium(II) Using Flow Injection

Mohammad A. Abdalla, Ibrahim Z. Al-Zamil, Saad A. Al-Tamrah, Telal S. Omar

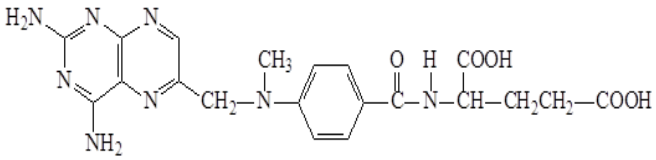
***Abstract*— A novel electro-generated chemiluminescence method for the determination of methotrexate (2,4-diamino-N^{10}-methyl pteroyl glutamic acid) in pharmaceutical formulations is proposed. The method was based on the chemiluminescence (CL) emission intensity produced as a result of the electrochemical oxidation of the $Ru(bpy)_3^{2+}$ into the active $Ru(bpy)_3^{3+}$ form, which then reacts with the methotrexate and produces light.**

Reaction variables were thoroughly investigated. The optimum conditions were incorporated in the procedure. Linear calibration curve were obtained for signal in mV versus concentration in mol L^{-1} in the range 0-21×10^{-7} M with percentage relative standard deviation of less than 2% (n = 6) and correlation coefficient of r = 0.99986. The method described here proved to be very convenient and easy to use for the assay of methotrexate in drug formulations. This method was tested by the determination of methotrexate in different drugs containing known concentration.

***Index Terms*— Flow injection electro-generated chemiluminescence, methotrexate (MTX), Tris(2,2'-bipyridyl) ruthenium(II) reagent.**

I. INTRODUCTION

Methotrexate (2,4-diamino-N^{10}-methyl-pteroglutamic acid, is widely used for the treatment of cancer. Vast number of reports have shown that it is highly effective in the treatment of Leukemia and other types of cancer such as Osteosarcoma, Choriocarcinoma and non-Hodgkin's Lymphoma. Its action is the shutting down of multiplication of cells [1,2].



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Several methods of determination of methotrexate have been reported. These methods including enzymatic assay [3], fluorescence [4], spectrometry [5-9], chromatography [10,11] and electrophoresis [12]. The development of high performance liquid chromatography and electrophoresis have the potential to detect both the intact molecule and its metabolities. However, these techniques require considerable instruments and expensive columns and long time for sample preparation and analysis. In this paper, we report an electro-generated chemiluminescence (ECL) for the determinations of methotrexate in pharmaceutical preparations. Sensitivity and linearity of the method will be discussed and our result is going to be compared with chemiluminescent method.

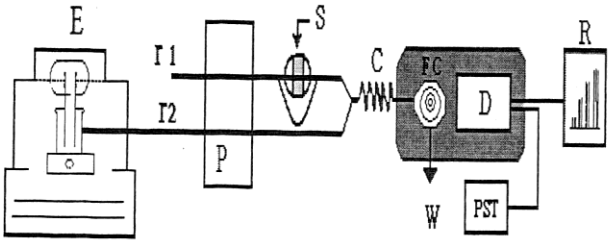

Figure 1.Schematic diagram of FIA-ECL system for ECL determination of methotrexate.
E = Electrical cell, r_1& r_2 = Reagent, S = Sample, C = coil, D = PMT, R = Recorder.

To the best of our knowledge, no chemiluminescence method has been yet used for the determination of methotrexate in pharmaceutical preparations. The proposed method describes a highly sensitive way for the determination of methotrexate using flow injection analysis-electro-generated chemiluminescence (CFIA-ECL) system (Fig. 1). The ECL method is based on the reaction of 2,2'-bipyridyl-ruthenium(II) and methotrexate as the chemical reductant.

II. EXPERIMENTAL

2.1 Materials and Reagents

All chemicals used in this work were of analytical grade (AnalaR) and all solutions were prepared using distilled water throughout. A solution of 1×10^{-2} M Tris(2,2'-bipyridyl) dichloro ruthenium(II) hexahydrated, $Ru(bpy)_3^{2+}$ (Aldrich, USA) was prepared in 0.05 M

sulfuric acid (DBH Ltd, UK). Methotrexate standard material was supplied from (Sigma, UK).

2.2 Instrumentation

The CL and ECL measurements were made with home built flow-injection cell. Potentials were applied to the two platinum electrodes.

In the case of ECL, one electrode is oxidizing $Ru(bpy)_3^{2+}$ and at the same time reduction of $Ru(bpy)_3^{2+}$ occurs at the surface of the other electrode. Enough period of time was allowed for the oxidation of $Ru(bpy)_3^{2+}$to $Ru(bpy)_3^{3+}$. Samples were then mixed with $Ru(bpy)_3^{3+}$ solution producing CL just before the photomultiplier detector. Measurements of the emitted radiation intensity were made by a sensitive photomultiplier tube (PMT) (Thorn EMI, 9789 QB).

The PMT used were operated at 1100 V, provided by a stable high voltage power supply (Thorn EMI, model PM 28BN), and so wavelength selection was involved. A 4-channel peristaltic pump (Gilson Miniplus 3MP4) was used to deliver the reagent and the samples.

A solenoid activated rotary valve (Rheodyne 5020) was used to inject the sample solution into reagent stream of 1×10^{-3} M $Ru(bpy)_3^{2+}$ in 0.05 M H_2SO_4. Polytrifluoroethylene (PTFE) of 0.06 mm internal diameter was used throughout the manifold and a strip-chart recorder RECIII, (Amersham Pharmacia Biotech) was fed to the PMT and the height of the peak was measured manually.

2.3 Standard solution

A stock solution containing 0.01 g of methotrexate in 100 ml (100 g ml^{-1}) was prepared.

2.4.1 General procedure

The reagent solution used for the electro-generated chemiluminescence [1.0×10^{-3} M. $Ru(bpy)_3^{3+}$ in 0.05 M sulfuric acid] was fed by the peristaltic pump at a flow rate of 4.0 l min^{-1}. Oxidation of $Ru(bpy)_3^{2+}$ to $Ru(bpy)_3^{3+}$ was care using platinum electrodes that is held at a voltage of 0.8 V for a peroxide of 80 min.

Working standard solutions of methotrexate were prepared from the stock solution in the range 0.1×10^{-3} 10×10^{-3} M. A 25 ml portion of each methotrexate solution was injected into a cancer stream of reagent solution and the resulting peak heights were recorded. Calibration graphs were prepared by plotting ECL/CL intensities (mV) versus the sample molar concentrations. Accordingly, the corresponding regression equations were calculated.

a) *2.5 Optimization of experimental variables*

For the analysis of methotrexate in pharmaceutical preparations ten powdered tablets equivalent to 10 mg of methotrexate were transferred into 100 ml volumetric flask and completed to volume with distilled water. The flask with its contents was sonicated for 5 minutes and then filtered.

In order to the medium used for the electro-generated chemiluminescence reaction to fulfill certain criteria, reaction variables were thoroughly investigated. In each time, changing one variable in every turn and keeping the others at their optimum conditions

2.5.1 Effect of the oxidation potential of $Ru(bpy)_3^{2+}$ reagent

The applied potential used for the oxidation of $Ru(bpy)_3^{2+}$ reagent was investigated in the range between (0.2-1.4 V). The highest ECL emission was obtained at 0.80 V which was used for the determination.

2.5.2 Effect of sulfuric acid concentration as a carrier stream of $Ru(bpy)_3^{2+}$

Oxidation potential of $Ru(bpy)_3^{2+}$ is increased by the use of sulfuric acid as a solvent. The effect of sulfuric acid concentration (0.01-2 M) on the ECL signal was investigated. It was found that ECL signal increases with increasing sulfuric acid concentration and the highest signals were obtained with 0.5 M sulfuric acid. Thus, 0.5 M acid concentration was used in the preparation of the reagent throughout methotrexate determination.

2.5.3 Effect of $Ru(bpy)_3^{2+}$ concentration

The effect of the reagent concentration on the signal of methotrexate was studied using concentrations in the range 1×10^{-4}-5×10^{-3} M $Ru(bpy)_3^{2+}$ prepared in 0.5 M sulfuric acid. It can be seen clearly from (Fig. 2) that the ECL signals were increased with increasing of $Ru(bpy)_3^{2+}$ concentration and the highest intensity of the signal was observed at the concentration 4×10^{-3} M $Ru(bpy)_3^{2+}$ after this concentration the signal height start decreasing. This may result due to quenching of ECL signal by means of self-absorption of emitted radiation.

2.5.4 Effect of reagent flow rate

After optimizing the concentrations of the reagents, attention were then concentrated on the effect of the reagents flow rate. The flow rate is an essential parameter and its variation has the greatest influence on the light of the signal obtained. The effect of flow rate was studied after keeping all other parameters constant, i.e. the solution of $Ru(bpy)_3^{2+}$ of 0.004 M in 0.5 M sulfuric acid was then introduced into the manifold at different solution flow rate. Increasing the flow rate tend to increase the signal height up to 2.2 ml min^{-1} after which it start to decrease. This flow rate is fairly high enough and therefore was chosen to enable the excited reaction product to reach the detector in a minimum time. Therefore, 2.2 ml min^{-1} was chosen as the best flow rate. At this flow rate, the consumption of the reagent is the minimum.

2.5.5 Effect of the sample volume

The effect of the sample volume in the range of 10-100 l was investigated by changing the length of the sample loop connected to the injection valve. It was found that the signal height of ECL of methotrexate increase sharply with the increase of the sample volume between 10-15 l. Concentration after that 50-100 ml of methotrexate tend to increase the peak height of ECL slightly. Therefore, 50 l was chosen as the optimum sample volume for a complete reaction without more consumption of the analyte.

2.5.6 Effect of the reaction coil length

The effect of the length of the reaction coil on the ECL signal height was studied in the range 10-150 cm. (Fig. 2) show the effect of the reaction coil length on the ECL intensity. When the coil length is taller than 40 cm, the height of the signal start decreasing notably most probably

because the reaction completes away from the PMT. Therefore, 40 cm length were chosen throughout this study.

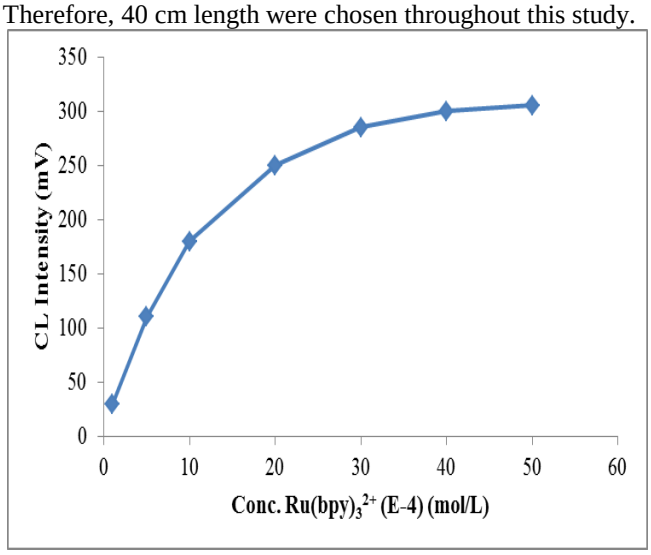

Figure 2.Effect of concentration of on methotrexate determination (15 g ml^{-1}).

III. VALIDATION

3.1.1 Linearity range

The calibration graph for the determination of methotrexate by the proposed method was made by plotting the ECL intensity versus the concentration of methotrexate. The linearity of calibration graph was found to be over the concentration range 0-21×10^{-7} M. The calibration graph was found to be recliner over the concentration range as seen in Tab. 1 and Fig. 3.

Table 1.Percentage analytical recovery.

Pharmaceutical preparation	Concentration ×10^{-7}mol l^{-1}*		Recovery (%)
	Taken	Found	
Methotrexate Tablets (Each tablet contains MTX equivalent to MTX 2.5 mg)	3.0	3.006	100.21
	7.0	7.010	100.14
	11.0	10.926	99.33
	15.0	14.970	99.80
	19.0	19.083	100.44
Mean ± S.D.	99.98±0.44		

* mean of three readings.

3.1.2Accuracy and precision

To check the accuracy of the proposed method percentage error was determined over the concentration range 0-21×10^{-7} M. The % error was recorded in Tab. 1. Table 2 shows the statistical evaluation of the proposed method.

Table 2.Statistical analysis for FIA-ECL determination of methotrexate.

Parameter	Short range
Linear range	0.18-168 ppm
Regression equation	y = 10.67 x + 13.262
Correlation coefficient	0.9998
Standard deviation of intercept	0.033
Standard deviation of slope	0.0014
LOD	0.018 ppm
LOQ	0.18 ppm

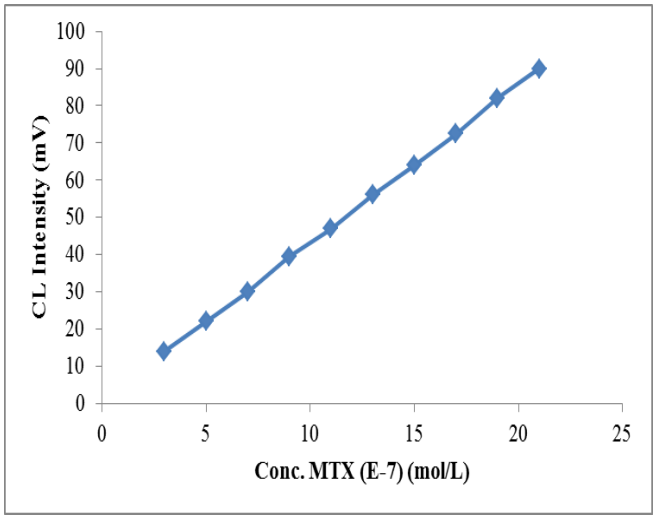

Figure 3.Calibration curve.

3.1.3Ruggedness

In order to evaluate the ruggedness of the method, the intraday and interday precisions were evaluated as is seen from Tab. 3. The precision of the proposed ECL method was fairly high, as indicated in the table by low values of relative standard deviation.

Table 3.Precision data for the determination of methotrexate by the proposed FIA-ECL method.

Parameter	Methotrexate concentration					
	Short range (ppm)			Long range (ppm)		
Found	2.5	5	10	10	20	30
	100.34	100.5	99.4	100.4	100.2	99.9
	99.8	99.3	100.3	100.2	100.4	99.4
Mean	100.2	99.6	99.6	100.1	100.3	99.7
%RSD	0.73	0.60	0.81	0.80	0.91	0.88

Each result is the average of six separate experiments.

3.4Applications

3.4.1 Analysis of methotrexate

The proposed method has showed a high sensitivity which allowed the determination of methotrexate in pharmaceutical preparations. The nominal content of methotrexate in the pharmaceutical preparation or methotrexate tablets was determined using the corresponding regression equation. Tab. 4 shows the obtained result. A statistical comparison of the proposed method with the CL method is shown in Tab. 5. The t-test value obtained indicate that there is no significant difference between the two methods, however, the calibration graphs of the ECL has the advantages over the CL of being superior when sample solution of lower concentration are to be analyzed.

Table 4.Determination of Methotrexate in some pharmaceutical preparation.

Pharmaceutical preparation	Concentration ×10^{-7} M		Recovery %
Methotrexate tablets Each tablet contains methotrexate equivalent to MT × 2.5 mg	3.0	3.006	100.21
	7.0	7.01	100.14
	11.0	10.926	99.33
	15.0	14.970	99.80
	19.0	19.083	100.44
Mean ± SD	99.98±0.44		

Table 5. Comparison of results obtained for the determination of methotrexate using the proposed FIA-ECL and FIA-CL methods

Pharmaceutical preparation	% Found ± RSD	
Methotrexate tablets Each tablet contains methotrexate equivalent to MT × 2.5 mg	FIA-CL	FIA-ECL
	99.82±0.51	99.98±0.44
f-values (6.59)	1.12	1.5
t-values (2.365)	1.56	1.09

3.4Electro-generated chemiluminescenct mechanism

The proposed mechanism is as follows: $Ru(bpy)_3^{2+}$ is oxidized electrochemically at the surface of the platinum electrodes at the appropriate voltage. The methotrexate is reduced to form an intermediate ion radical which is reacts with the $Ru(bpy)_3^{2+}$ to form $Ru(bpy)_3^{+}$. This is then reduces $Ru(bpy)_3^{3+}$ to the excited state that emits light. The proposed mechanism of methotrexate may be shown as follows:

$Ru(bpy)_3^{2+} \longrightarrow Ru(bpy)_3^{3+}$ at the surface of the electrode.

$Ru(bpy)_3^{3+}$ + methotrexate $\longrightarrow$ methotrexate$^{+\cdot}$ + $Ru(bpy)_3^{2+}$

$Ru(bpy)_3^{2+}$ + methotrexate$^{+\cdot}$ + $H_2O \longrightarrow$ methotrexate + H^+ + $Ru(bpy)_3^{+}$

$Ru(bpy)_3^{+}$ + $Ru(bpy)_3^{3+} \longrightarrow Ru(bpy)_3^{2+}$ + $Ru(bpy)_3^{2+}$

$Ru(bpy)_3^{2+} \longrightarrow Ru(bpy)_3^{2+}$ + h□

IV. CONCLUSION

Our proposed Flow Injection-Electro-generated Chemiluminescence (FI-ECL) has proven to be suitable and sensitive for the determination of methotrexate in pharmaceutical preparations. The oxidation of $Ru(bpy)_3^{2+}$ is selective, that means lower concentration of methotrexate will not be oxidized as in the case of chemiluminescence utilizing potassium permanganate which otherwise oxidize the drug itself.

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